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Exhibit G

USE OF ANTISENSE OLIGONUCLEOTIDES TARGETING THE ANTIAPOPTOTIC GENE, CLUSTERIN/TESTOSTERONE-REPRESSED PROSTATE MESSAGE 2, TO ENHANCE ANDROGEN SENSITIVITY AND CHEMOSENSITIVITY IN PROSTATE CANCER

MARTIN E. GLEAVE, HIDEAKI MIYAKE, TOBY ZELLWEGER, KIM CHI, LAURA JULY, COLLEEN NELSON, AND PAUL RENNIE

ABSTRACT

Background: Androgen resistance develops, in part, from upregulation of antiapoptotic genes after androgen withdrawal. Identification and targeting of genes mediating androgen-independent (AI) progression may lead to development of novel therapies that delay hormone-refractory prostate cancer. Clusterin is a cell survival gene, that increases after androgen ablation. Here, we review clusterin's functional role in apoptosis and the ability of antisense oligonucleotides (ASOs) against clusterin to enhance apoptosis in prostate cancer xenograft models.

Results: Immunostaining of radical prostatectomy specimens confirm that clusterin is highly expressed in 80% prostate cancer cells after neoadjuvant hormone therapy, but is low or absent (<20%) in untreated specimens. Clusterin levels increase >10 fold in regressing Shionogi tumors after castration. Pretreatment of mice bearing androgen-dependent Shionogi tumors with calcium antagonists inhibited castration-induced apoptosis, tumor regression, and clusterin gene upregulation, illustrating that clusterin is an apoptosis-associated gene and not an androgen-repressed gene. Clusterin ASOs reduced clusterin levels in a dose-dependent and sequence-specific manner. Adjuvant treatment with murine clusterin ASOs after castration of mice bearing Shionogi tumors decreased clusterin levels by 70% and resulted in earlier onset and more rapid apoptotic tumor regression, with significant delay in recurrence of AI tumors. Species-specific clusterin ASOs also increased the cytotoxic effects of paclitaxel, reducing the 50% inhibitory concentration (IC_{50}) of PC-3 and Shionogi cells by 75% to 90%. Although clusterin ASOs had no effect on the growth of established AI Shionogi or PC-3 tumors, clusterin ASOs synergistically enhanced paclitaxel-induced tumor regression in both Shionogi and PC-3 models.

Conclusions: Collectively, these data identify clusterin as an antiapoptosis protein, upregulated in an adaptive cell-survival manner by androgen ablation and chemotherapy, which confers resistance to various cell-death triggers. Inhibition of clusterin upregulation using clusterin ASOs can enhance cell death after treatment with androgen ablation and chemotherapy. UROLOGY **58** (Suppl 2A): 39–49, 2001. © 2001, Elsevier Science Inc.

Androgen withdrawal is the only effective form of systemic therapy for men with advanced disease, producing symptomatic and/or objective response in 80% of patients. Unfortunately, androgen-independent (AI) progression and death occurs within a few years in most of these cases.[1] Prostate cancer is highly chemoresistant, with objective response rates of 10% and no demonstrated survival benefit.[2] More recently, phase 2 studies using taxane-based combination regimens are reporting objective responses in 20% to 30% and prostate-specific antigen (PSA) responses in >50% of cases.[3–5] Hormone-refractory prostate cancer (HRPC) is therefore the main obstacle to improving the survival and quality of life in patients with advanced disease, and novel therapeutic strategies

From the Prostate Centre, Vancouver General Hospital; and Division of Urology, University of British Columbia, Vancouver, British Columbia, Canada

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Martin E. Gleave is a patent inventor for clusterin ASO.

Reprint requests: Martin E. Gleave, MD, Division of Urology, University of British Columbia, D-9, 2733 Heather Street, Vancouver, British Columbia V5Z 3J5, Canada

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that target the molecular basis of androgen and chemoresistance are required. Traditionally, new nonhormonal therapies are evaluated late in HRPC, and when used in this end-stage setting, most are ineffective.[2] A more rational treatment strategy would involve adjuvant therapy, which targets adaptive changes in gene expression precipitated by androgen withdrawal to enhance castration-induced apoptosis (eg, Bcl-2, clusterin) and to delay emergence of the AI phenotype.

Progression to androgen independence is a complex process involving variable combinations of clonal selection,[6] adaptive upregulation of antiapoptotic survival genes,[7–10] androgen-receptor transactivation in the absence of androgen from mutations or increased levels of coactivators,[11,12] and alternative growth factor pathways, including HER-2/neu, epidermal growth-factor receptor, and insulin-like growth factor I.[12–14] It is interesting and somewhat ironic to note that the very same agents used to kill or control cancer cells also trigger cascades of events that lead to a chemoresistant phenotype. If we are to have a significant impact on survival, improved understanding of specific mechanisms mediating AI progression and new therapeutic strategies designed to inhibit the emergence of this phenotype are necessary.

Insights and understanding into the nature of human prostate cancer will come from developing useful concepts through careful extrapolation from appropriate types of tumor models.[15] Several appropriate tumor model systems have been developed, including the human cell lines LNCaP,[16,17] LuCaP,[18] CRW22,[19] LAPC9,[12] and the murine Dunning[6] and Shionogi.[9] The human LNCaP cell line is androgen responsive, PSA secreting, and immortalized in vitro.[16] Similar to clinical prostate cancer, serum PSA levels in the LNCaP tumor model are initially regulated by androgen, are directly proportional to tumor volume, and increase after prolonged periods of growth after castration to signal progression to androgen independence.[17] Apoptotic tumor regression does not consistently occur after castration, but tumor growth stabilizes, and serum and tumor-cell PSA levels decrease by 80% for several weeks after castration, after which LNCaP tumor growth rates and PSA expression increase above precastrate levels.

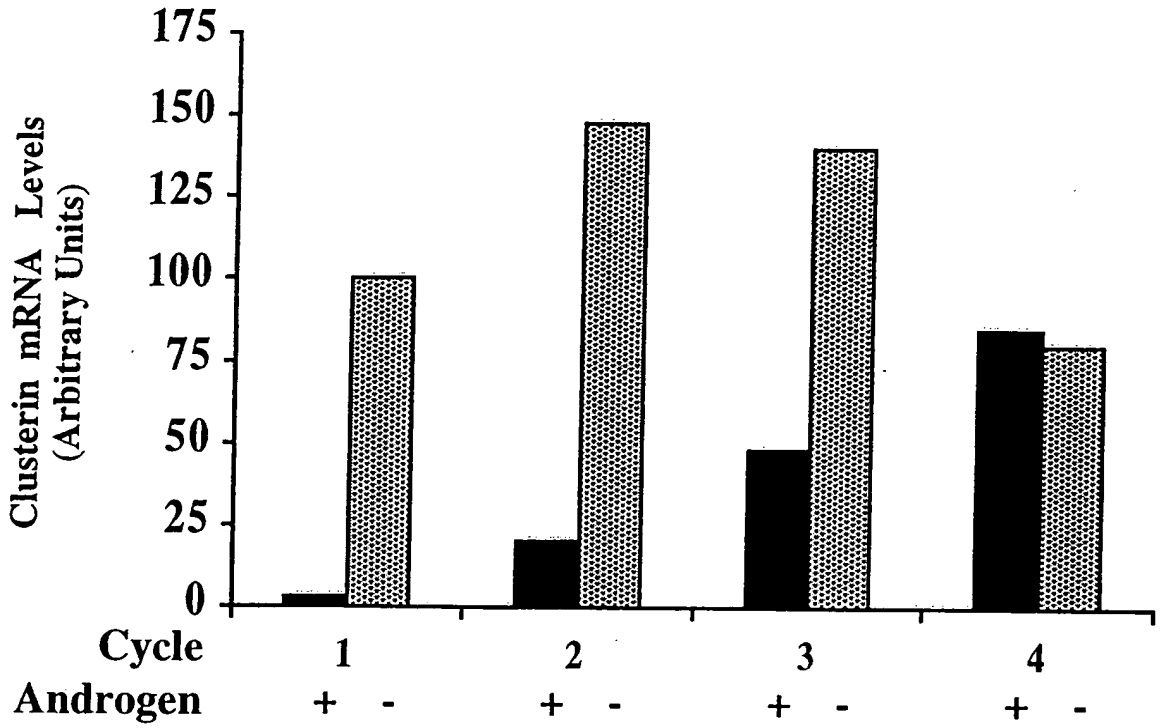
The Shionogi tumor model is a mouse androgen-dependent (AD) mammary carcinoma that, as with human prostate cancer, regresses after castration and later recurs as AI tumors.[9] Although the Shionogi tumor model is of mouse mammary origin, it shares a number of features characteristic of human prostate cancer in that it is AD, has a functional androgen receptor, and undergoes extensive castration-induced apoptosis after androgen withdrawal with subsequent AI tumor recurrence after 1 month. The pattern of changes in gene expression after castration in the LNCaP and Shionogi models are similar to human prostate cancer (eg, Bcl-2, clusterin, insulin-like growth factor–binding proteins, PSA, Bcl-x_L). Collectively, these similarities validate their use as models of the human disease for functional genomics and preclinical proof of principle experiments.[7,8,10,20–23]

Investigations using various tumor models demonstrate that hormonal resistance and chemoresistance develops, in part, from alterations in the apoptotic machinery, because of increased activity of antiapoptotic pathways or expression of antiapoptotic genes. Research during the past decade has identified several proteins that may promote progression and resistance by inhibiting apoptosis. Of special relevance to development of AI progression and HRPC are those survival proteins upregulated after apoptotic triggers (eg, androgen ablation), which function to inhibit cell death. Proteins fulfilling these criteria include antiapoptotic members of the Bcl-2 protein family and clusterin. We[24] and others[25–27] have reported that Bcl-2 levels increase after androgen withdrawal and during AI progression, and that Bcl-2 ASOs enhance cancer cell death after treatment with androgen withdrawal or chemotherapy.[22,28,29] In this review, we will summarize the role of the apoptosis-associated protein, clusterin, in the development of androgen resistance and chemoresistance.

CLUSTERIN AS AN INHIBITOR OF APOPTOSIS

Also known as testosterone-repressed prostate message 2 (TRPM-2), or sulfated glycoprotein 2, clusterin is associated with a wide variety of physiologic and pathologic processes,[30] including Alzheimer's disease,[31] renal diseases, such as renal dysplasia, membranous glomerulonephritis, gentamicin nephrotoxicity, ureteric obstruction, and inherited polycystic renal diseases.[32] Clusterin is associated with numerous tumors including prostate,[33] lung,[34] breast,[35,36] lymphoma,[37] and renal cell carcinoma.[38] Clusterin levels increase dramatically during castration-induced apoptosis in rat prostate epithelial cells,[39] in AD Shionogi tumors,[8] and human prostate cancer CRW22,[23] and PC-82[40] xenografts. Furthermore, clusterin levels increase and decrease during each cycle of intermittent androgen suppression in the Shionogi tumor model (Figure 1).

Although clusterin was first isolated >15 years ago, its function has remained elusive. Although its functional relation to apoptosis has been intensively investigated, considerable ambiguity remains on roles for clusterin as both a mediator of



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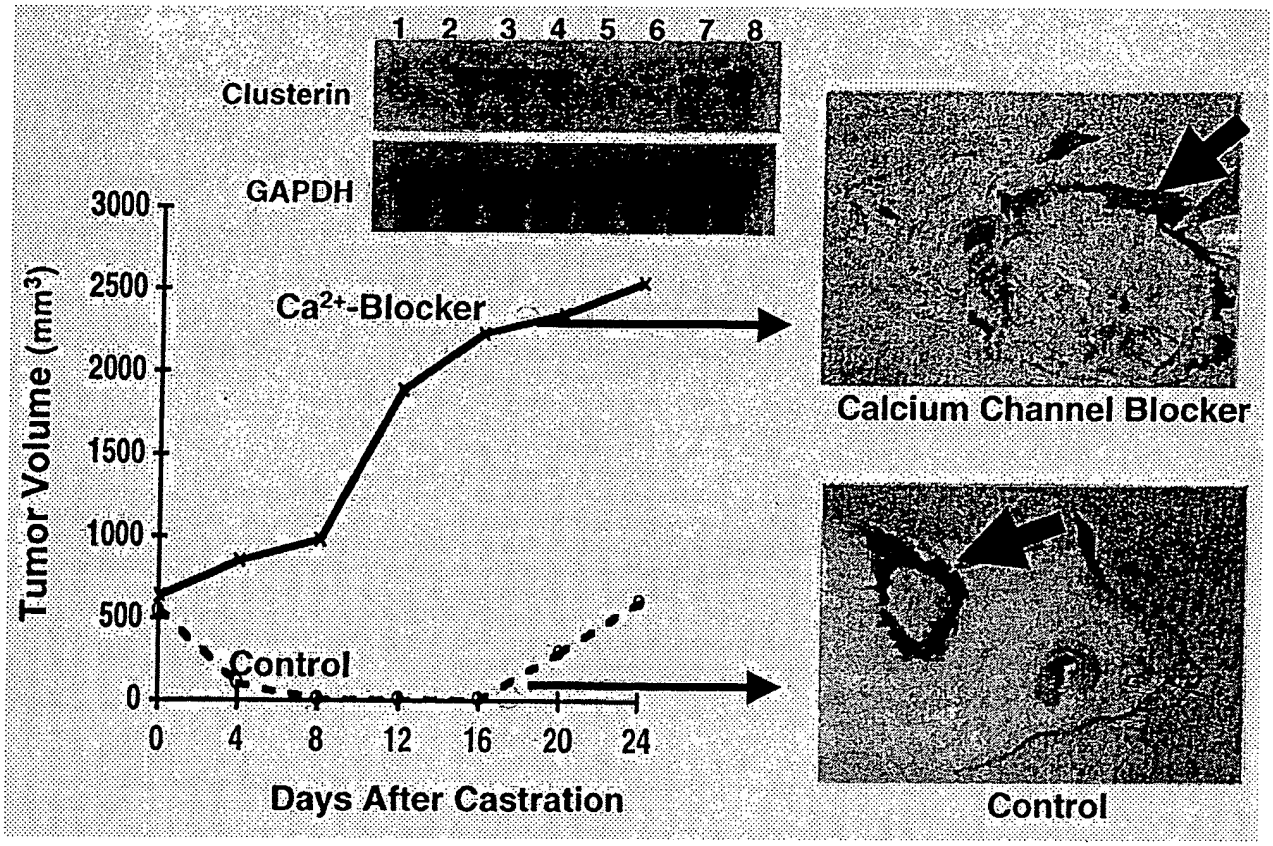
apoptosis and as a cell survival factor. In the prostate gland, clusterin messenger RNA (mRNA) was originally cloned as TRPM-2 from regressing rat prostate, where it was subsequently shown by in situ hybridization to be expressed in dying epithelial cells.[39] Subsequently, however, clusterin was shown to be an apoptosis-associated, rather than an androgen-repressed gene; clusterin upregulation did not occur when calcium antagonists were used to inhibit castration-induced apoptosis and Shionogi tumor regression (Figure 2).[8] Support for clusterin having an active role in promoting apoptosis is largely derived implicitly from its direct association with a great variety of dying tissues, such that measurement of clusterin is an accepted marker of apoptotic cell death.[41–47]

Unraveling the function of clusterin has been an elusive goal; it has been ascribed many and sometimes contradictory functions. For example, nuclear clusterin has recently been shown to interact with Ku-70 and act as a cell-death signal in MCF-7 breast cancer cells transfected with various green-fluorescing protein–tagged clusterin constructs.[47] In contrast, the preponderance of recent studies on the role of clusterin in apoptosis provide strong evidence in support of a cell survival and antiapoptotic function (for reviews, see references 48 and 49) Because clusterin binds to a wide variety of biologic ligands[48–50] and is regulated by transcription factor heat shock factor 1,[51] an emerging view suggests that clusterin functions as heat shock protein to chaperone and stabilize conformations of proteins at time of cell stress. Some investigators suggest that clusterin may be the first-identified, secreted mammalian chaperone, but the precise site(s) of action remains unknown.[49] Although clusterin is constitutively secreted, and extracellular clusterin has a cytoprotective function,[52] proapoptotic signals may induce intracellular forms through differential translation.[50]

Although emerging data identify clusterin as an inhibitor of apoptosis, little is known about mechanisms and location of clusterin action at the molecular and cellular level. The seemingly paradoxical roles for clusterin in apoptosis are perhaps analogous to those ascribed to 2 forms of Bcl-x that arise from alternative splicing.[53–55] The smaller form, Bcl-x_S, can act to inhibit the protective effects of the larger form Bcl-x_L, as well as Bcl-2 through an unknown mechanism. Similarly, clusterin can exist in many molecular forms, although they apparently arise from posttranslational modifications of a single mRNA transcript.[56] Furthermore, using a panel of antibodies directed against various α- and β-chain epitopes of clusterin, Lakins *et al.*[57] were able to immunologically distinguish the



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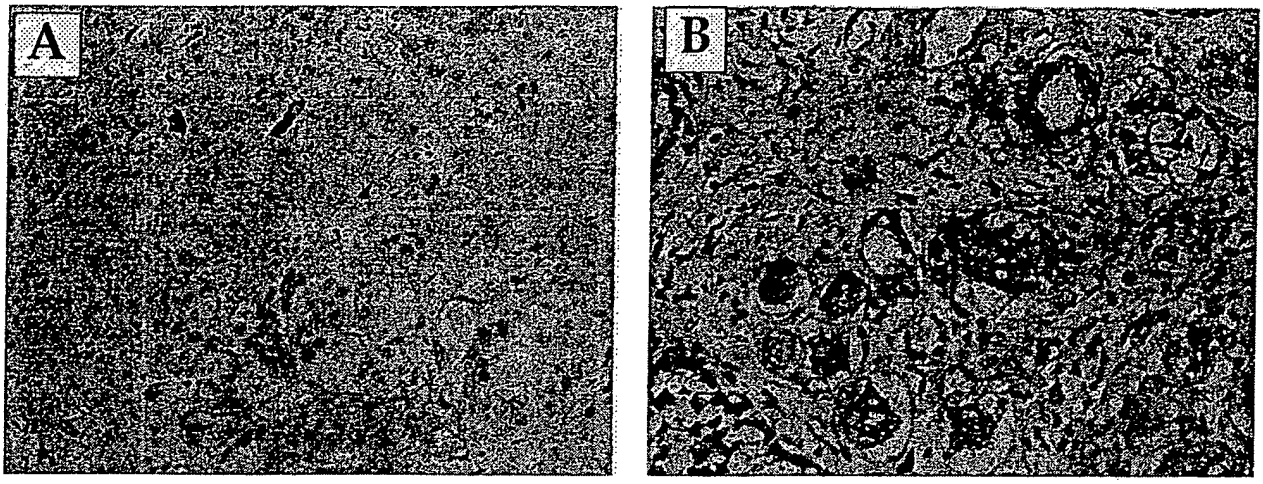
forms of clusterin upregulated in dying cells from those synthesized by surviving cells in regressing rat prostate. The secreted mature and core proproteins are believed to interact with glycoprotein 330, a cell surface receptor.[58] Collectively, these reports suggest that pro- and antiapoptotic functions of clusterin may be because of activity of different forms of the protein.

Clusterin as a Therapeutic Target in Prostate Cancer

In prostate cancer, experimental and clinical studies show clusterin expression is associated with AI progression and has a protective role against apoptotic cell death. For example, introduction of TRPM-2 complementary DNA (cDNA) into LNCaP prostate cancer cells increases resistance to apoptosis induced by tumor necrosis factor α treatment.[59] Increased expression of clusterin in prostate cancer is closely correlated with higher Gleason scores.[33] Residual foci of cancer cells from radical prostatectomy specimens treated with neoadjuvant androgen ablation exhibited strongly positive staining for clusterin. Clusterin staining in specimens from neoadjuvantly treated patients increased in intensity (+3 to +4) in 80% to 90% of cancer cells compared with absent (0)-to-low intensity staining (+1 to +2) in 10% to 20% of cancer cells in nonneoadjuvant hormonal therapy (NHT)-treated specimens (Figure 3). Clusterin levels also increase in prostate and other cancer cells after chemotherapy and radiation (Figure 4).

Clusterin Overexpression Confers a Resistant Phenotype

To investigate the functional significance of clusterin upregulation after androgen withdrawal, the effects of clusterin overexpression on time to AI progression after androgen ablation was evaluated by stably transfecting LNCaP cells with TRPM-2 cDNA expression vector. Tumor volume and serum PSA levels increased 4-fold faster after castra-



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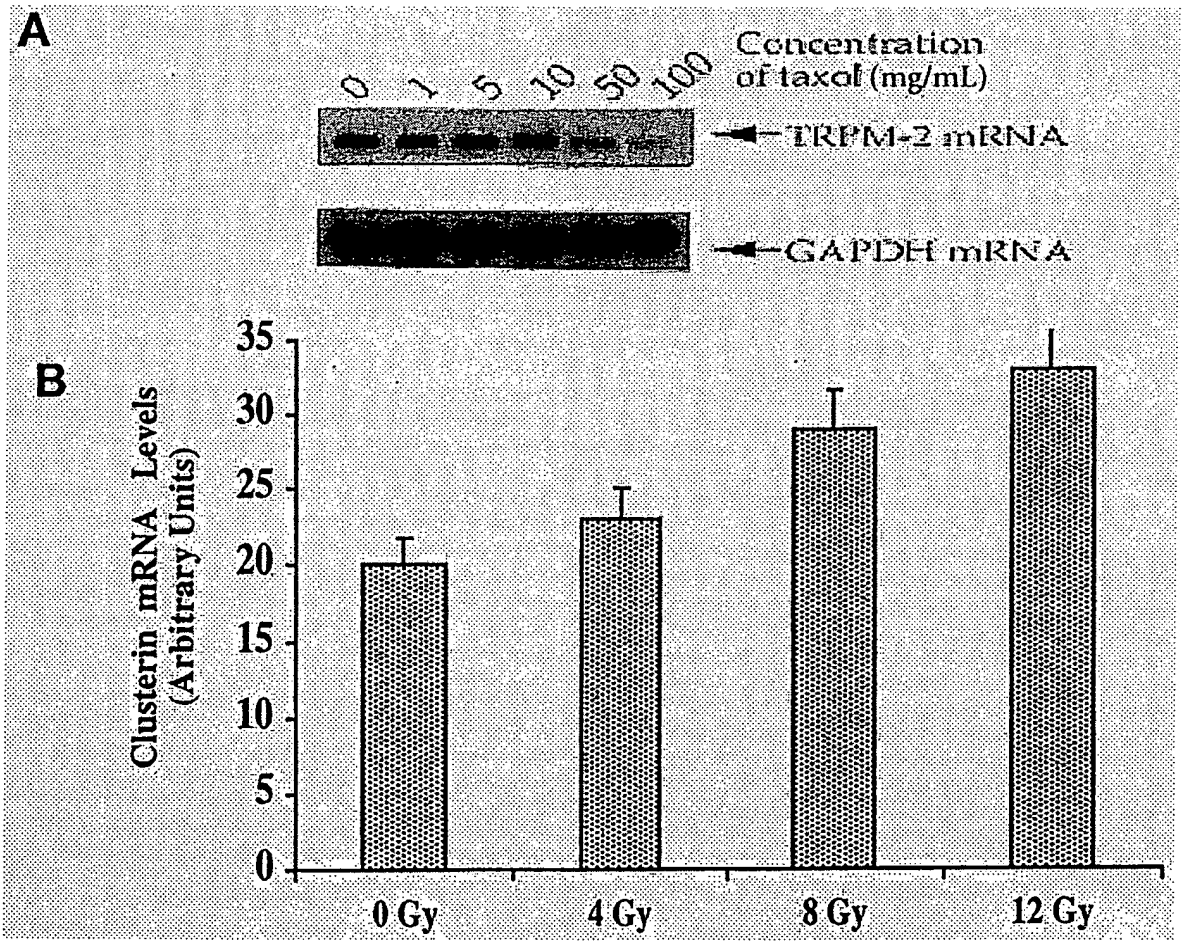

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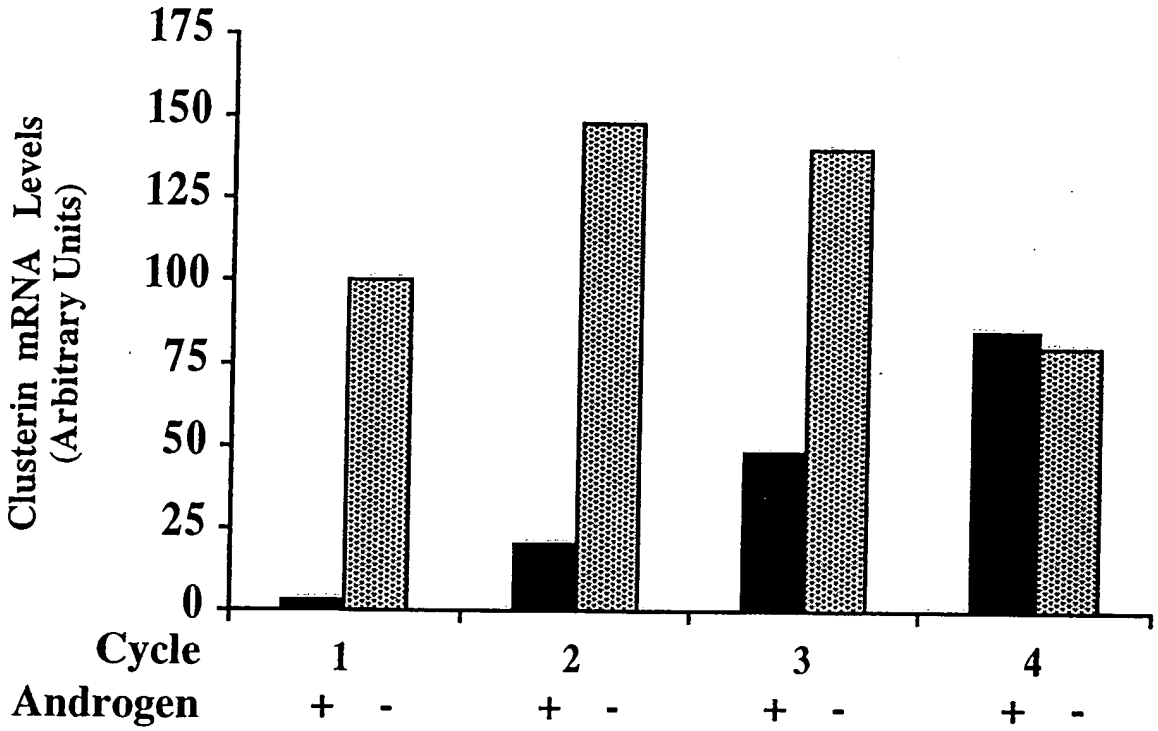

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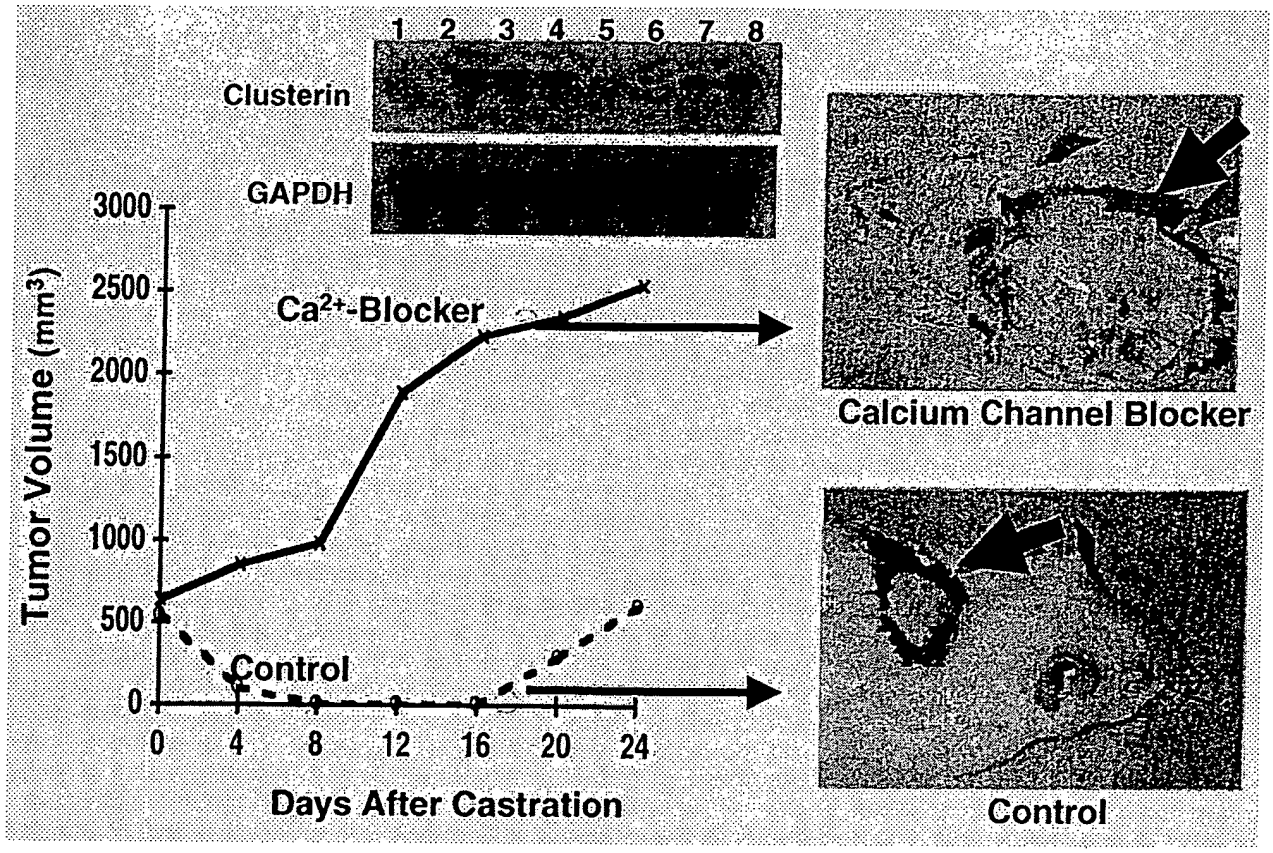

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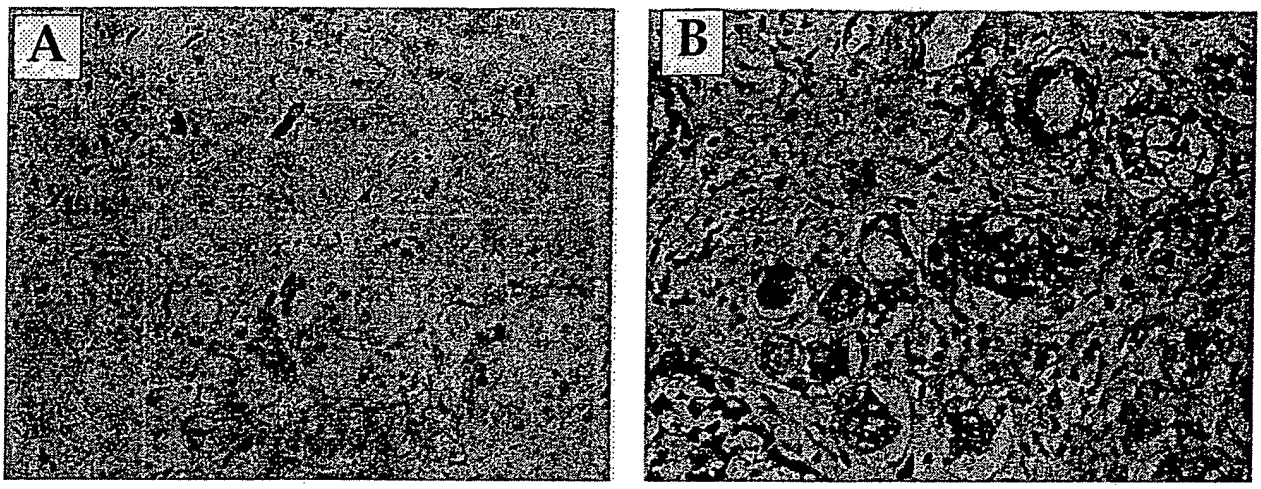

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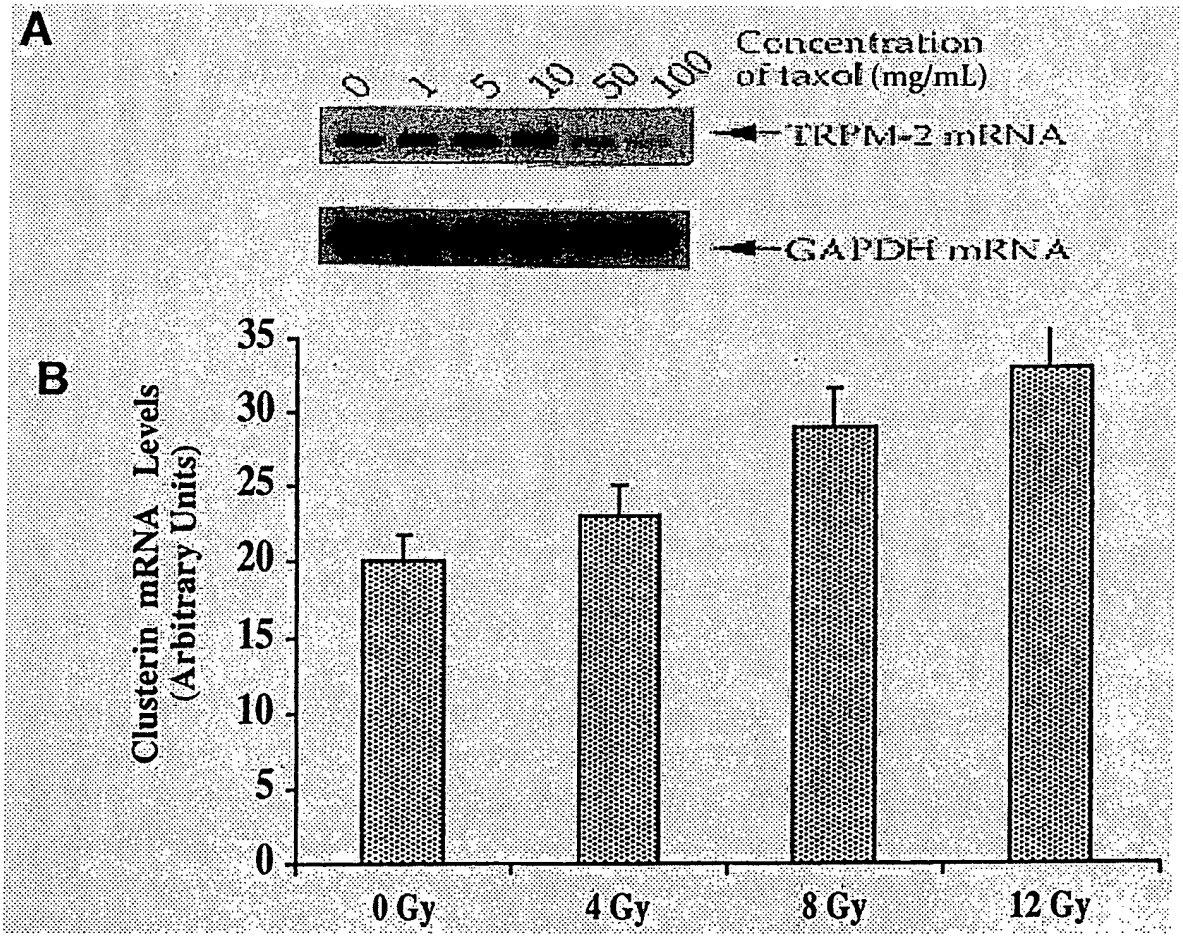

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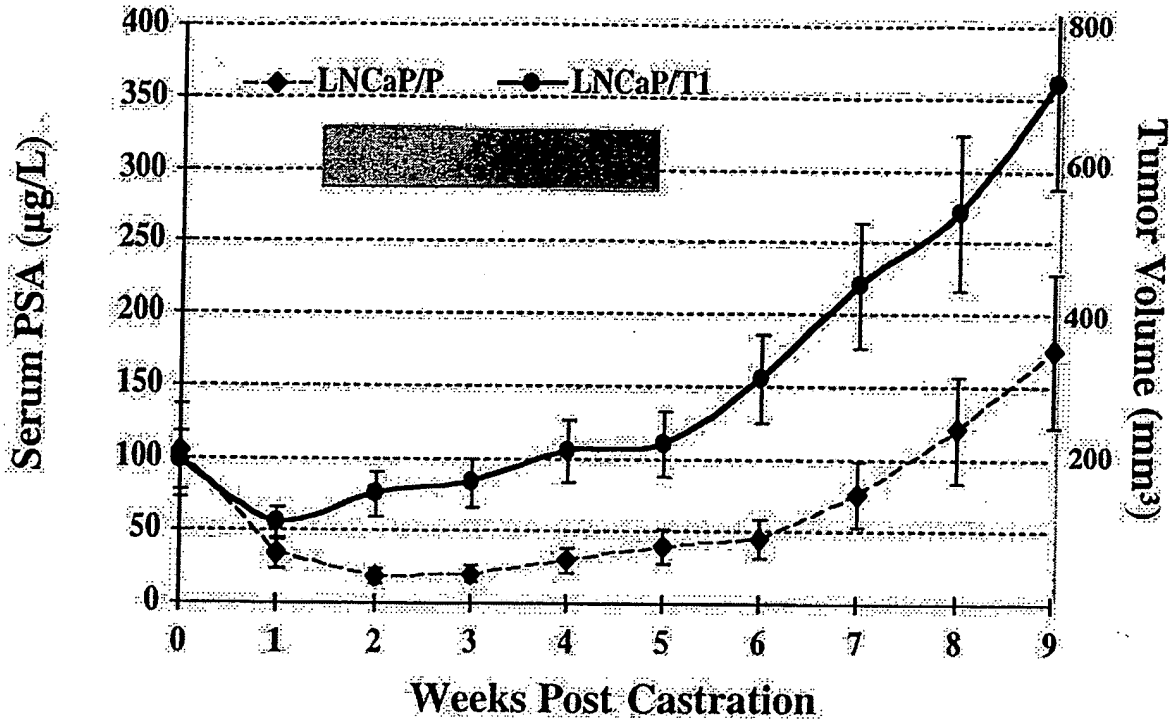

FIGURE 5. *Overexpression of clusterin accelerates time to AI progression in the LNCaP tumor model. LNCaP cells were transfected with TRPM-2 cDNA expression vector pRC-CMV/TRPM-2 or the pRC-CMV vector alone as a control. As shown in inset, abundant levels of clusterin mRNA were detected in clusterin-transfected clones (LNCaP/T), whereas the parental LNCaP (LNCaP/P) or control vector–transfected cell line (LNCaP/C) did not express detectable TRPM-2 mRNA levels. Intact male nude mice were inoculated subcutaneously with 1 million LNCaP/C and LNCaP/T1 cells. After castration, LNCaP/T tumor volume and serum PSA levels continued to increase after castration, increasing 3-fold faster compared with controls. AI = androgen independent; cDNA = complementary DNA; mRNA = messenger RNA; PSA = prostate-specific antigen; TRPM-2 = testosterone-repressed prostate message 2.*

tion in clusterin-overexpressing LNCaP/T1 tumors compared with control tumors (Figure 5).[8] Furthermore, overexpressing clusterin LNCaP/T1 tumors were more resistant to paclitaxel and radiation than control tumors.[60] These findings demonstrate that clusterin is a cell survival gene upregulated by apoptotic triggers (as with androgen withdrawal, chemotherapy, radiation) and confers resistance when overexpressed.

Enhancing Hormone Sensitivity using Clusterin Antisense Oligonucleotides

Targeting cell survival genes upregulated by androgen withdrawal may enhance castration-induced apoptosis and thereby prolong time to overt recurrence. ASOs are chemically modified stretches of single-strand DNA, complementary to mRNA regions of a target gene, which inhibit translation by forming RNA/DNA duplexes, thereby reducing mRNA and protein levels of the target gene.[61] The specificity and efficacy of ASOs rely on precise targeting afforded by strand hybridization, where only a perfect match between the target sequence and the ASOs will lead to hybridization and inhibition of translation. Phosphorothioate ASOs are water soluble, stable agents manufactured to resist nuclease digestion through substitution of a nonbridging phosphoryl oxygen of DNA with sulfur. ASOs become associated with high-capacity, low-affinity serum-binding proteins after parental administration.[62] ASOs targeting several oncogenes have been reported to specifically inhibit expression of these genes and delay progression in several types of tumors.[63–65] We[7,22] and others[28] have reported on the use of ASOs to specifically target the cell survival gene, Bcl-2, to enhance hormone and chemotherapy. A limiting factor of phosphorothioate backbone is its short, in vivo half-life, necessitating continuous infusion for human clinical trials. A 2′-O-(2-methoxy)ethyl (MOE) ribose modification has been shown to increase both binding affinity and resistance of ASOs.[66] Because of its longer tissue half-life, MOE-modified ASOs may be amendable to weekly administration scheduling.

ASOs corresponding to the clusterin translation initiation site reduce clusterin levels in a dose-dependent and sequence-specific manner.[8,64] AD Shionogi tumors regressed faster, and complete regression occurred earlier after castration in mice

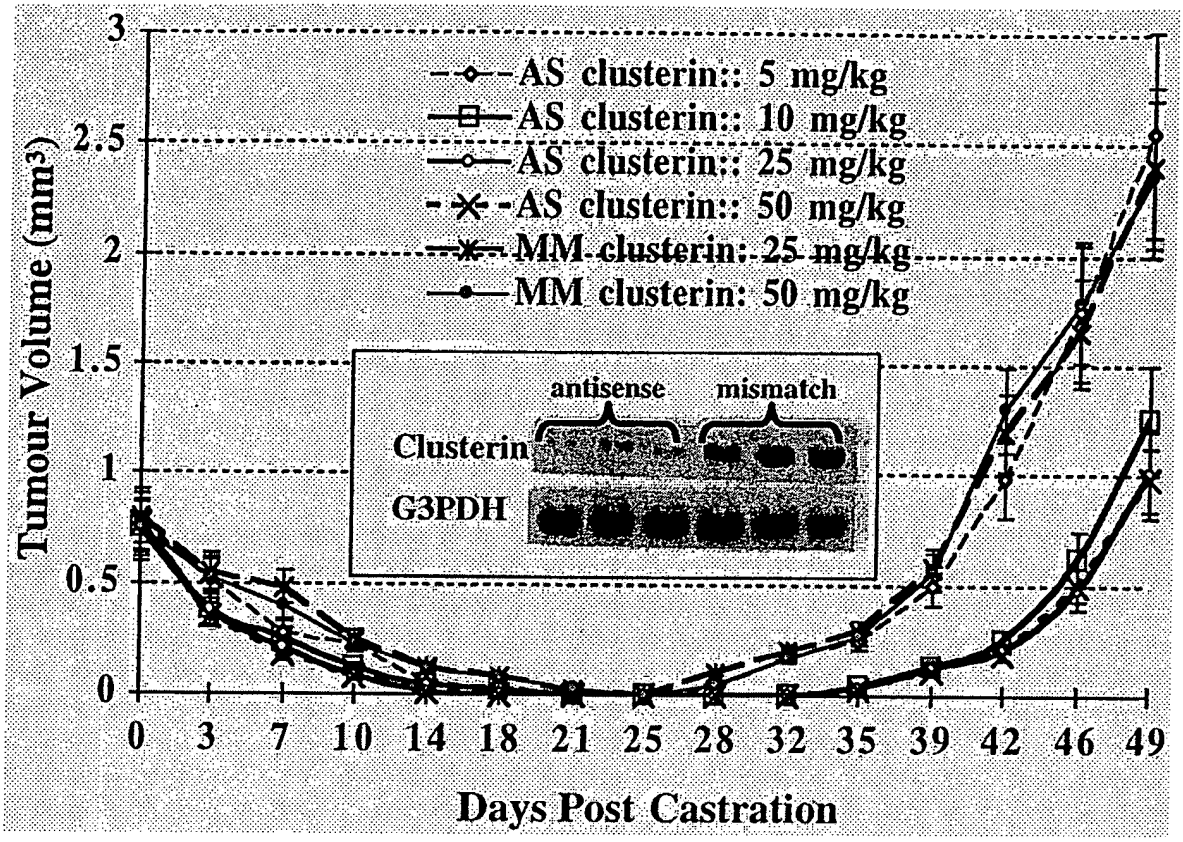

FIGURE 6. *Dose-dependent effects of clusterin antisense oligonucleotides (ASOs) on Shionogi tumor growth. Beginning on the day of castration, 5 to 50 mg/kg ASO or mismatch (MM) control oligonucleotide was injected intraperitoneally once daily for 15 days into each mouse bearing Shionogi tumors. Tumor volume was measured twice weekly and calculated by the formula length × width × depth × 0.5236. Each point represents the mean tumor volume in each experimental group containing 7 mice with standard deviations. (*Inset*) Northern analysis shows reduced clusterin levels from tumors harvested on day 3 after castration plus ASO treatment.*

treated with clusterin ASO compared with controls. Furthermore, clusterin ASO treatment significantly delayed recurrence of AI tumors; tumor volume in the mismatch-treated control group was 6 times greater than the ASO-treated group by day 50 postcastration.[8] Dose-dependent studies using mouse clusterin ASOs in the murine Shionogi model demonstrated dose-dependent activity ≤25 mg/kg per day, but did not reveal any obvious toxicity in mice treated with ≤50 mg/kg per day (Figure 6).

Enhancing Chemosensitivity using Clusterin Antisense Oligonucleotides

Clusterin ASOs also increased the cytotoxic effects of mitoxanthrone and paclitaxel, reducing the 50% inhibitory concentration (IC_{50}) of PC-3 and Shionogi cells by 75% to 90%.[60,64] The induction of apoptosis by 10 nmol/L taxol, as demonstrated by DNA laddering, and poly adenosine diphosphate ribose polymerase cleavage could be seen only when used with clusterin ASOs (Figure 7). Although clusterin ASOs had no effect on the growth of established AI Shionogi or PC-3 tumors, clusterin ASOs synergistically enhanced paclitaxel-induced tumor regression in both the Shionogi and human PC-3 models.[60,64]

Clusterin may also play a role in mediating chemoresistance in renal cell carcinoma and other tumors. We recently reported that clusterin levels are significantly higher in renal cell carcinoma compared with adjacent normal renal tissues.[67] Pretreatment of Caki-2 cells with clusterin ASOs decreased clusterin levels and significantly enhanced chemosensitivity to paclitaxel in vitro. Characteristic apoptotic DNA laddering occurred after combined treatment with clusterin ASO plus paclitaxel, but not with either agent alone. In vivo administration of clusterin ASO synergistically enhanced paclitaxel-induced Caki-2 tumor regression and delayed tumor progression by 50%.

Enhancing Radiation Sensitivity Using Clusterin Antisense Oligonucleotides

Little is known about the molecular mechanisms that contribute to intrinsic radioresistance characteristic of prostate cancer. Bcl-2–overexpressing LNCaP cells appear to be more resistant to radia-

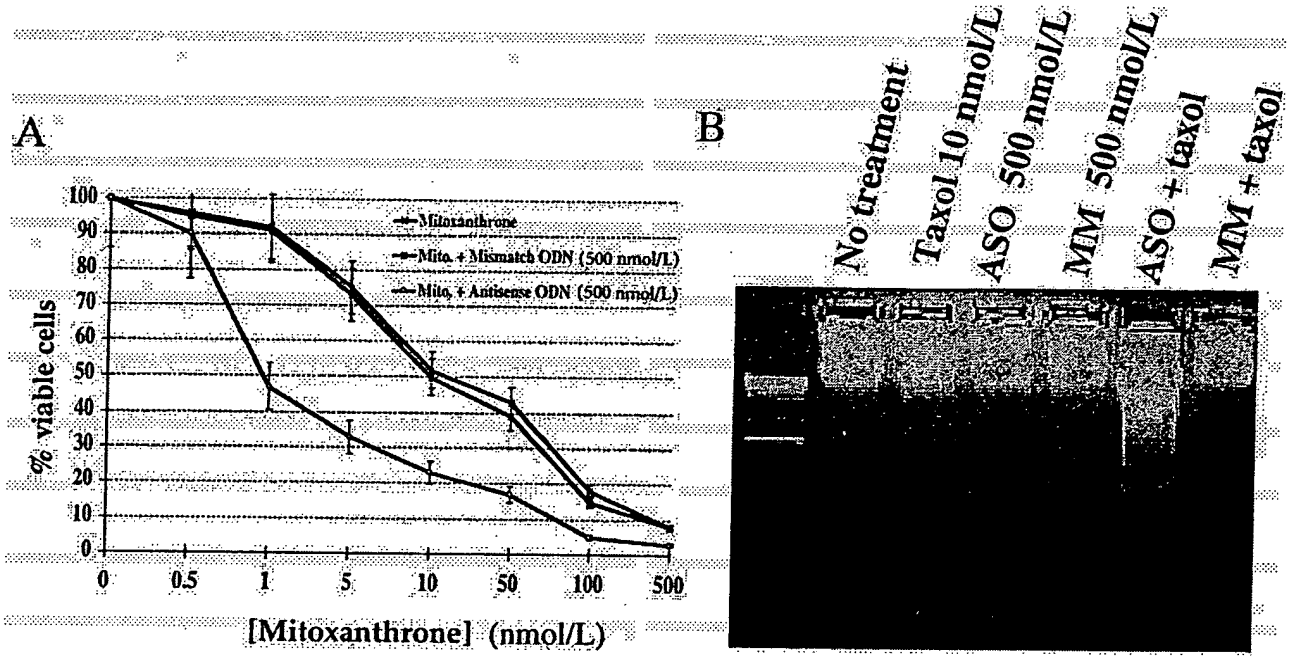

FIGURE 7. *Effect of combined treatment with clusterin antisense oligonucleotides (ASO) plus chemotherapy on Shionogi tumor cell growth. (A) Shionogi tumor cells were treated daily with 500 nmol/L clusterin ASO or mismatch control oligonucleotide for 2 days. Cells were then treated with medium containing various concentrations of mitoxanthrone. After 48 hours of incubation, cell viability was determined by tetrazolium dye (MTT) assay. (B) Shionogi cells were treated daily with 500 nmol/L clusterin ASO or mismatch (MM) control oligonucleotide for 2 days, and treated with 10 nmol/L paclitaxel. After 48 hours of incubation, DNA was extracted from culture cells, electrophoresed in a 2% agarose gel, and visualized by ethidium bromide staining and ultraviolet transillumination. DNA laddering indicates apoptosis only in cells treated with combined paclitaxel and clusterin.*

tion-induced apoptosis and tumorigenesis compared with parental cells.[68] Similarly, clusterin-overexpressing LNCaP cells were less sensitive to irradiation with significantly lower cell-death rates (23% after 8 Gy) compared with parental LNCaP cells (50% after 8 Gy) 3 days after irradiation.[69] Clusterin expression in PC-3 cells after radiation increased in a dose-dependent manner in vitro by 70% up to 12 Gy and in vivo by >80%. Inhibition of clusterin expression in PC-3 cells using clusterin ASOs before radiation significantly decreased PC-3 cell growth rate and plating efficiency, and enhanced radiation-induced apoptosis. In vivo administration of clusterin ASO before and after radiation significantly reduced PC-3 tumor volume by 50% at 9 weeks as compared with mismatch control oligonucleotides. These findings support the hypothesis that clusterin acts as a cell survival protein that mediates radioresistance through the inhibition of apoptosis.

SUMMARY

The data reviewed above identify clusterin as an antiapoptosis protein, upregulated in an adaptive cell survival manner by androgen ablation and chemotherapy, which confers resistance to various cell-death triggers, including hormone therapy, radiation therapy, and chemotherapy. Inhibition of clusterin upregulation using clusterin ASOs can enhance cell death after treatment with androgen ablation and chemotherapy. A clinical trial in men with HRPC using clusterin ASOs in combination with docetaxel is planned to begin in late 2002.

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DISCUSSION FOLLOWING DR. MARTIN E. GLEAVE'S PRESENTATION

Ronald Lieberman, MD (Rockville, Maryland): If you give these compounds chronically, is there a suggestion that you eventually develop either resistance or progression? Just in your own system, if you give the bcl-2, do you get an upregulation of the TRPM-2? Is there cross-talk there?

Martin E. Gleave, MD (Vancouver, British Columbia, Canada): Those are questions that the array technology is going to allow us to answer. Ultimately, all these tumors progress. All we are doing is pushing the recurrence curve. But if you can delay progression, then, especially in an elderly population with prostate cancer, you may be able to buy them time to die of something else along the way.

Dr. Lieberman: My understanding is that in prostate cancer there is overexpression of whatever the gene family is for mul-